Strand invasion by mixed base PNAs and a PNA–peptide chimera

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ABSTRACT

Peptide nucleic acid oligomers (PNAs) have a remarkable ability to invade duplex DNA at poly-purine–polypyrimidine target sequences. Applications for PNAs in medicine and biotechnology would increase if the rules governing their hybridization to mixed base sequences were also clear. Here we describe hybridization of PNAs to mixed base sequences and demonstrate that simple chemical modifications can enhance recognition. Easily synthesized and readily soluble eight and 10 base PNAs bind to plasmid DNA at an inverted repeat that is likely to form a cruciform structure, providing convenient tags for creating PNA–plasmid complexes. PNAs also bind to mixed base sequences that cannot form cruciforms, suggesting that recognition is a general phenomenon. Rates of strand invasion are temperature dependent and can be enhanced by attaching PNAs to positively charged peptides. Our results support use of PNAs to access the information within duplex DNA and demonstrate that simple chemical modifications can make PNAs even more powerful agents for strand invasion. Simple strategies for enhancing strand invasion should facilitate the use of PNAs: (i) as biophysical probes of double-stranded DNA; (ii) to target promoters to control gene expression; and (iii) to direct sequence-specific mutagenesis.

INTRODUCTION

Peptide nucleic acids (PNAs) are a DNA/RNA mimic in which the phosphate deoxyribose backbone has been replaced by uncharged *N*-(2-aminoethyl)glycine linkages (1,2). Hybridization by PNAs is not hindered by electrostatic repulsion and is characterized by high melting temperatures (3) and rates of association (4). The uncharged PNA backbone is unlikely to interact with cellular proteins that normally bind negatively charged macromolecules (5,6), a distinct advantage for specific recognition of targets within cells relative to recognition by oligomers with phosphodiester or phosphorothioate linkages.

One of the most remarkable properties of PNAs is their ability to recognize sequences within duplex DNA by strand invasion. Early studies demonstrated that polypyrimidine PNAs invaded target DNA duplexes by forming a four-stranded complex consisting of a PNA–PNA–DNA triplex and a displaced DNA strand (1,7). Subsequent experiments revealed that strand invasion can be improved by use of bis-homo-pyrimidine PNAs (8). Mechanistic studies by Frank-Kamenetskii and colleagues have suggested that this complex is initiated by formation of a triplex between one PNA and the DNA duplex and that subsequently strand invasion occurs (9). Strand invasion also occurs at inverted repeats (4,10) and the recent observation of double duplex formation by pseudo-complementary PNAs containing diaminopurine–thiouracil base pairs (11) suggests that PNA recognition can be made even more general. Published applications for strand invasion by PNAs include purification of genomic DNA (12,13), cleavage of plasmid DNA by PNA–nuclease conjugates (10), cleavage of rare sequences within genomic DNA (14), artificial activation of transcription (15,16), inhibition of transcription (17–19), targeted oligonucleotide hybridization (PD-loop formation) (4,20), site-directed mutagenesis of cellular DNA (21) and labeling of plasmids with fluorophores (22).

Here we examine strategies for enhancing strand invasion by PNAs. While polypurine–polypyrimidine sequences are a common feature within DNA, discovery of the rules governing hybridization to a broader range of sequences would increase the potential of PNAs as tools for manipulating nucleic acid structure. We describe the synthesis and hybridization of PNAs directed to several different sequences within a supercoiled plasmid and simple chemical modifications that improve recognition.

MATERIALS AND METHODS

Synthesis of oligonucleotide–peptide conjugates, PNAs and PNA derivatives

PNAs were obtained through automated synthesis using an Expedite 8909 synthesizer (PE Biosystems, Foster City, CA), using the manufacturer's protocols, and were analyzed by mass spectral analysis as described (23). PNA monomers and other reagents for PNA synthesis were obtained from PE Biosystems. Conjugates between PNAs and biotin or peptides were synthesized and analyzed as described (23). 5′-Thiol-modified oligonucleotides were activated for disulfide exchange using 2,2′-dithiodipyridine as described (4). The synthesis of oligo-nucleotide–peptide conjugates was performed as described (4)

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using disulfide exchange to conjugate 5′-S-thiopyridyl-containing oligonucleotides with peptides containing an N-terminal cysteine.

Promotion of strand elongation by PNAs

Supercoiled plasmid pUC19 DNA (24) (σ = 0.5) was prepared by a mild lysis protocol followed by two successive CsCl gradient ultracentrifugations to minimize the likelihood of contamination by denatured or nicked duplex DNA (25). Hybridization of conjugate and plasmid was accomplished by mixing pUC19 (40 nM) with 20 equivalents of oligonucleotide–peptide conjugate in 10 mM Tris–HCl, pH 7.5, buffer for 15 min at 37°C. The hybridized primer–template mixture was then cooled on ice and MgCl$_2$, NaCl and Tris–HCl, pH 7.5, were added to final concentrations of 8, 80 and 10 mM, respectively. The labeling mix consisting of modified T7 DNA polymerase (Sequenase; US Biochemical, Cleveland, OH) (1 U/reaction) and [^{35}S]dATP (Amersham) were added and DNA sequencing using bound peptide–oligonucleotide as primer was carried out. Equal volumes of the elongation reactions were applied to a denaturing 6% polyacrylamide gel and were separated by electrophoresis. The products were visualized by autoradiography and quantified using a Molecular Dynamics (Sunnyvale, CA) model 425F phosphorimager.

To examine the effects of PNA hybridization on strand elongation by oligonucleotide–peptide conjugates, five equivalents of PNA or PNA derivatives were mixed with pUC19 at temperatures between 37 and 50°C for 1 h. After these incubations, the PNA/plasmid mixtures were cooled on ice to 0°C and treated with 10 equivalents of oligonucleotide–peptide conjugate for 30 min. In all cases the oligonucleotide–peptide conjugates are complementary to the PNA strand. Incubations were performed at 0°C because at this temperature the displaced strand remains accessible to hybridization but direct strand invasion by the oligonucleotide–peptide conjugate is prevented.

The absolute level of hybridization was estimated by analyzing the elongated primer–template complex by agarose gel electrophoresis and examining the shift in mobility of the plasmid and the incorporation of radioactivity (4). The percentage of plasmid bound to PNA was estimated by the following procedure. Using altered plasmid mobility as analyzed by agarose gel electrophoresis, an oligonucleotide–peptide conjugate directed to the inverted repeat at bases 1542–1562 has been shown to hybridize to virtually 100% of the plasmid. This hybridization was repeated for every experiment to provide a positive control for the experimental procedure and a standard for evaluation of hybridization efficiency. The radiolabel incorporated during priming by the oligonucleotide–peptide conjugate at bases 1542–1562 was compared to the radiolabel incorporated by the PNA–template–primer complex being tested, providing a lower limit for the percentage of bound plasmid.

Affinity capture of biotin–PNA conjugates

Affinity capture of plasmid DNA employed Dynabeads M-280 derivatized with streptavidin (Dynal, Oslo, Norway) as a matrix for separation of plasmids bound to biotin-labeled PNAs. pUC19 (40 nM) was mixed with biotin-labeled PNAs (500 nM) in 10 mM Tris–HCl, pH 8.0, at 37°C for 30 min prior to addition of streptavidin-coated beads. Varying concentra-
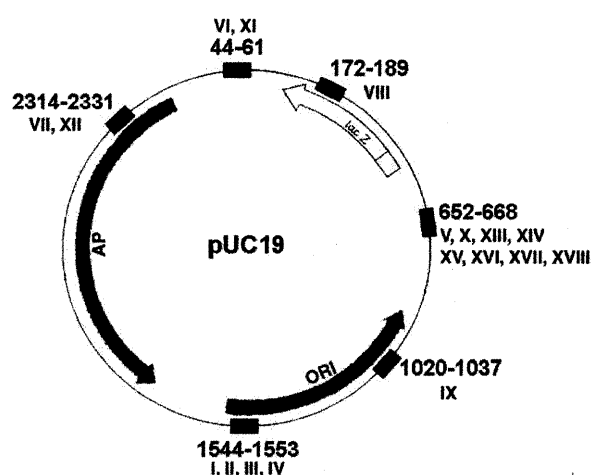

Figure 1. Location of the target sites for PNA hybridization within pUC19.

tions of sodium chloride, potassium chloride and magnesium chloride were added to the plasmid either before or after PNA addition as described. Beads, plasmid and PNA were incubated for 18 h at 22°C in 10 mM Tris–HCl, pH 8.0, 200 mM NaCl. The beads were then washed with 10 mM Tris–HCl, 1 mM EDTA, 200 mM NaCl to remove unbound plasmid. Bound plasmid was eluted from the beads by incubating at 80°C for 30 min in 10 mM Tris–HCl, 1 mM EDTA, 1 M NaCl. DNA in the supernatant was precipitated using ethanol and analyzed by 1% agarose gel electrophoresis.

RESULTS AND DISCUSSION

Binding of PNAs to an inverted repeat

Strand invasion by PNAs creates a displaced strand that is accessible to hybridization by DNA oligonucleotides, a phenomenon known as PD-loop formation (20). Modified T7 DNA polymerase can extend the hybridized oligonucleotide and the resulting polymerization products provide a convenient marker for monitoring strand invasion. We have previously shown that 18 base PNA **I** can hybridize to a sequence within supercoiled plasmid DNA that contains an inverted repeat (Fig. 1) and facilitate formation of an active complex between template, primer and polymerase. Strand invasion by PNA **I** occurs despite the presence of a five base hairpin stem. While hybridization was efficient, the 18 base PNA was difficult to synthesize and relatively insoluble compared to PNAs of similar size and base composition, possibly because of the tendency for the stem to form intramolecular hydrogen bonds rather than be more fully solvated. To overcome these unfavorable properties and promote use of mixed base PNAs for strand invasion, we searched for simpler PNAs that can also bind inverted repeats. We compared sequence recognition by PNA **I** with recognition by 14 base PNA **II**, 10 base PNA **III** and eight base PNA **IV** (Table 1).

The use of PNAs to promote formation of an active complex between primer and template requires several steps (Fig. 2). The first step is incubation of PNA with plasmid at 37°C to

Table 1. Sequences and hybridization sites for PNAs and PNA–peptide chimeras

| PNA	Sequence	Site in pUC19	MS (found/calculated)
I	Biotin-LL-AGG ATC TAG GTG AAG ATC-Lys	1545–1562	5630.15/5628.90
II	AGG ATC TAG GTG AA-Lys	1549–1562	4031.75/4027.86
III	AGG ATC TAG G-Lys	1553–1562	2922.26/2919.80
IV	AGG ATC TA-Lys	1555–1562	2338.07/2337.23
V	Gly-CGC GCG GGG AGA GGC GG-Lys	652–668	4962.25/4961.57
VI	Gly-GCT CCC GGA GAC GGT CAC-Lys	44–61	5070.02/5066.80
VII	Gly-AAA CTC TCA GGG ATC TTA-Lys	2314–2331	5045.90/5047.89
VIII	TGA GAG TGC ACC ATA TGC-Lys	172–189	5049.59/5047.86
IX	CCT GTC CGC CTT TCT CCC-Lys	1020–1037	4841.62/4837.72
X	Pep1-Gly-CGC GCG GGG AGA GGC GG-Lys	652–668	6414.23/6413.63
XI	Pep1-Gly-GCT CCC GGA GAC GGT CAC-Lys	44–61	6517.04/6518.86
XII	Pep1-Gly-AAA CTC TCA GGG ATC TTA-Lys	2314–2331	6497.77/6499.95
XIII	Pep2-Gly-CGC GCG GGG AGA GGC GG-Lys	652–668	6510.07/6509.69
XIV	Pep1-Gly-CGC GCG GGG AGA GGC GG-Gly-Pep1	652–668	7796.87/7794.50
XV	Pep1-Gly-CGC GCG GtG AGA GGC GG-Lys	652–668	6384.85/6388.62
XVI	Pep1-Gly-CGC tCG GtG AGA tGC GG-Lys	652–668	6335.59/6338.63
XVII	Gly-CGC GCG GtG AGA GGC GG-Lys	652–668	4934.97/4936.57
XVIII	Gly-CGC tCG GtG AGA tGC GG-Lys	652–668	4883.31/4886.57

The sequence of peptide 1 (Pep1) is KK(AAKK)$_3$. The sequence of peptide 2 (Pep2) is KK(SSKK)$_3$. PNAs and peptides are listed from the N- to C-terminus. Mismatched bases in PNAs **XV–XVIII** are depicted in lower case and bold.

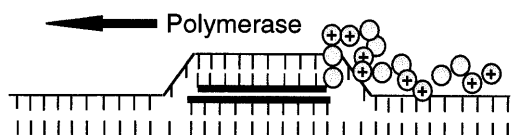**Step 1. Mix PNA and plasmid DNA to form PNA-plasmid complex**
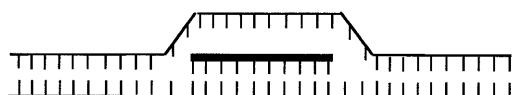
Step 2. Cool PNA plasmid complex to 0°C, a temperature that is not permissive for unaided binding of oligonucleotide-peptide conjugate

Step 3. Add oligonucleotide-peptide conjugate to form PNA/primer/template complex
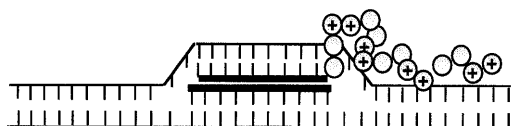
Step 4. Add modified T7 DNA polymerase and elongate primer

Polymerase

Step 5. Analyze strand elongation by polyacrylamide or agarose gel electrophoresis

Step 6. Estimate absolute efficiency of hybridization

Figure 2. Use of PNAs to facilitate formation of an active primer–template complex and subsequent strand elongation by modified T7 DNA polymerase.

allow binding. Once PNA binds, a DNA strand will be displaced, making it accessible to hybridization by a disulfide-linked conjugate between DNA and the cationic peptide C(AAKK)$_4$. This conjugate contains a free 3′-hydroxyl and can act as a primer for modified T7 DNA polymerase. A cationic peptide is attached to the 5′-termini of the DNA to increase the rate of hybridization and make the assay more rapid and quantitative. The second step requires lowering the temperature of the PNA/plasmid mixture to 0°C, a temperature at which the target sequence is not accessible in the absence of PNA and, after cooling, the DNA–peptide conjugate is added. The DNA–peptide conjugate is added after cooling because at low temperatures it cannot hybridize independently, making the observed signal dependent on PNA binding. The third step involves addition of modified T7 polymerase and nucleotides and standard DNA sequencing. The products of strand elongation are separated using denaturing PAGE and phosphorimager analysis is used to quantitate the relative efficiency of hybridization. The absolute efficiency of recognition is estimated by comparison to elongation by an oligonucleotide–peptide conjugate that hybridizes to almost 100% of the plasmid and represents a minimum estimate.

Creation of an active primer–template complex is a convenient method for evaluating strand invasion because it allows low levels of hybridization to be detected. The sequence information obtained confirms that the PNA is binding to its intended target site. We found that all four PNAs promoted formation of active primer–template complex (Fig. 3). Even the shortest PNA, eight base PNA **IV**, was able to form active
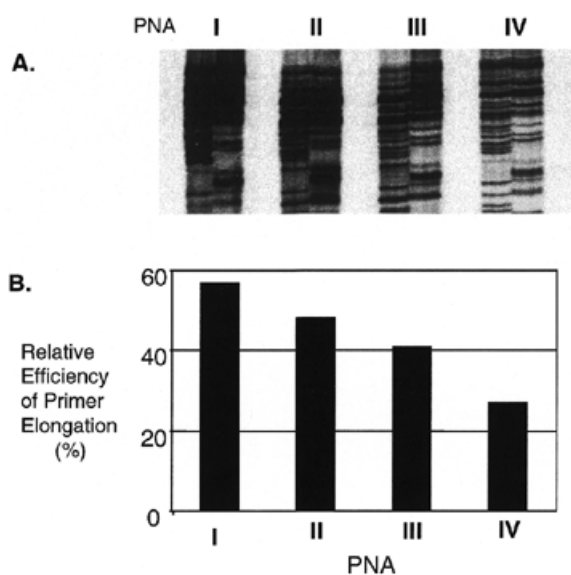
Figure 3. Recognition of an inverted repeat as a function of PNA length. PNAs **I** (18 bases), **II** (14 bases), **III** (10 bases) and **IV** (eight bases) were hybridized to pUC19 for 1 h, followed by primer addition and elongation as described in Materials and Methods. (**A**) Analysis of strand elongation of PNA–primer–template complex by polyacrylamide gel electrophoresis. Elongation reactions were terminated by addition of ddA (left lane in each pair) and ddT (right lane in each pair). (**B**) Quantitation of relative efficiencies of strand elongation by phosphorimager analysis. Efficiencies are relative to elongation by an oligonucleotide–peptide conjugate that hybridizes to ~100% of plasmid present and represent minimum estimates for the efficiency of hybridization of PNA to plasmid. Quantitation is based on triplicate measurements of strand elongation.

complex with ~20% of the plasmid. PNAs **II**, **III** and **IV** were much more soluble than PNA **I** and should afford more convenient agents for modifying plasmid DNA. The relative efficiencies of strand elongation correspond in rank order with the melting temperatures of PNAs **I**–**IV** (PNA **I**, 80°C; PNA **II**, 74°C; PNA **III**, 63°C; PNA **IV**, 49°C) for complementary DNA oligonucleotides.

An important practical consideration for applying strand invasion by PNAs as a tool for biotechnology is the effect of monovalent and divalent cations on hybridization. To explore this dependence, we used streptavidin-coated beads to capture complexes between the plasmid and PNAs labeled with biotin. We did not use our primer extension assay because high salt concentrations interfere with polymerization by modified T7 DNA polymerase and can prevent observation of hybridization. Hybridization of PNA **I** to the inverted repeat occurred at concentrations of sodium chloride as high as 200 mM, potassium chloride as high as 150 mM and magnesium chloride as high as 1 mM (Fig. 4A). Strand invasion at polypurine–polypyrimidine sequences is also very sensitive to the concentration of divalent cation and this observation is often cited as a barrier to strand invasion by PNAs within cells. Cells, however, present a much more complex environment for strand invasion and *in vivo* recognition can only be evaluated by directly testing the ability of PNAs to influence gene activation, repression or mutagenesis upon introduction into cells. Once hybridized, the bound PNA remained associated with the plasmid in the presence of up to 750 mM sodium chloride, 500 mM potassium chloride or

5 mM magnesium chloride (Fig. 4B). It is also important to note that we did not observe hybridization to non-supercoiled DNA substrates. This result is in accord with classic observations by Holloman *et al.* on D-loop formation by single-stranded DNA (26), previous work from our laboratory with PNAs and PNA–nuclease conjugates (10) and the observation by Bentin and Nielsen that supercoiling enhances strand invasion by polypyrimidine PNAs (27).

Strand invasion by PNAs to sequences that do not contain inverted repeats

Inverted repeats are atypical target sequences because they can form cruciform structures that offer highly accessible sites for the initiation of Watson–Crick base pairing. To evaluate the ability of PNAs to invade other sequences we compared hybridization of PNA **I** to hybridization of PNAs complementary to five sites that lack inverted repeats (**V**–**IX**) (Table 1). We observed that PNAs **V**, **VII** and **IX** could promote formation of an active primer–template while hybridization of PNAs **VI** and **VIII** yielded only minimal strand elongation (Fig. 5). None of the complementary oligonucleotide–peptide conjugates were able to promote strand elongation in the absence of PNA.

These results were surprising because we had previously observed that PNA **V** did not bind plasmid DNA (28). Thirteen of 17 bases in PNA **V** are purines and purine-rich PNAs are prone to aggregation (29). In the experiments we describe here, PNA solutions were briefly heated to 65°C to disrupt aggregates prior to incubation with DNA, and it is likely that this treatment accounts for the observed strand invasion. Homopurine PNAs are known to bind duplex DNA through strand invasion (30) and we speculate that the presence of nine consecutive purines within PNA **V** may compensate for the stability of the C/G-rich target and encourage the initiation of binding. It is interesting to note that the three PNAs showing the most efficient hybridization, **V**, **VII** and **IX**, all contain at least five consecutive purine or pyrimidine bases (**V**, CGCGCGGGGAGAGGCGG; **VII**, AAACTCTCAAGGATCTTA; **IX**, CCTGTCCGCCTTTCTCCC). This observation suggests that polypurine–polypyrimidine regions within PNAs can promote strand invasion even when they comprise only part of the PNA. The use of a triplex-forming DNA oligonucleotide to guide strand invasion has been observed by Gamper *et al.* (31) and it is possible that initial triplex formation is also directing strand invasion by PNAs **V**, **VII** and **IX**.

Enhanced recognition by PNA–peptide chimeras

The ability of PNAs to hybridize at sequences that are neither inverted repeats nor polypurine–polypyrimidine tracts provides a starting point for optimizing strand invasion. Previously, we had observed that attachment of a cationic peptide (AAKK)$_4$ to phosphodiester oligonucleotides increased the association rate constants for hybridization up to 48 000-fold (32). We reasoned that attachment of this peptide to PNAs might also enhance strand invasion. To test this hypothesis we synthesized PNA–peptide conjugates **X**, **XI** and **XII** containing peptide KK(AAKK)$_3$ to be analogous in sequence to PNA **V**, **VI**, and **VII**, respectively (Table 1). In each case, attachment of the KK(AAKK)$_3$ peptide doubled formation of the active primer–template complex (Fig. 6). We also synthesized conjugates **XIII** and **XIV** that were analogous in sequence to PNA **V** but contained peptide SS(SSKK)$_3$ or
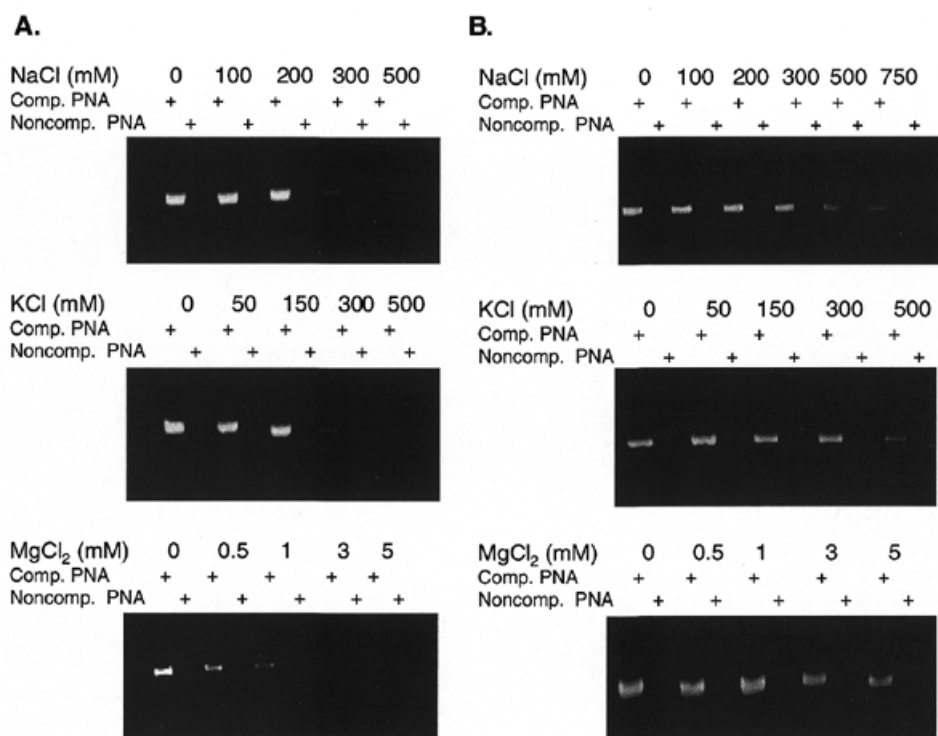
A.

B.

Figure 4. Effect of the concentration of sodium chloride, potassium chloride and magnesium chloride on binding of PNA **I**. Binding of biotin-labeled PNA **I** complementary to the inverted repeat at base pairs 1545–1562 within pUC19 and a control PNA that was not complementary to any sequence within pUC19 was carried out at 37°C for 30 min either before (**A**) or after (**B**) addition of the indicated monovalent or divalent salts. Complexes between plasmid and PNA were isolated by biotin–streptavidin affinity capture as described in Materials and Methods.
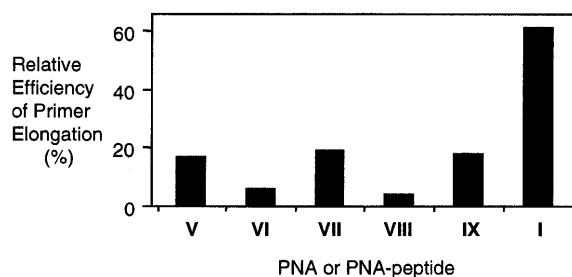

Figure 5. Formation of active primer–template complex as a function of target sequence within pUC19. Elongation products were separated by polyacrylamide gel electrophoresis and quantified by phosphorimager analysis. Efficiencies are relative to elongation by an oligonucleotide–peptide conjugate that hybridizes to ~100% of plasmid present and represent minimum estimates for the efficiency of hybridization of PNA to plasmid. Quantitation is based on triplicate determination of strand elongation.

were doubly derivatized with peptide KK(AAKK)$_3$ at both the N- and C-termini. PNA–peptide conjugate **XIII** promoted strand invasion and formation of primer–template complex with an efficiency similar to that of conjugate **X**, suggesting that the ability of serine to form additional hydrogen bonds does not affect conjugate association. Doubly modified conjugate **XIV** was more effective, yielding an additional 2-fold increase in the efficiency of formation of active primer–template complex and an overall 4-fold increase in recognition.

To characterize enhanced recognition by PNA–peptide chimeras we monitored recognition as a function of peptide modification, time and temperature (Table 2 and Fig. 7). At
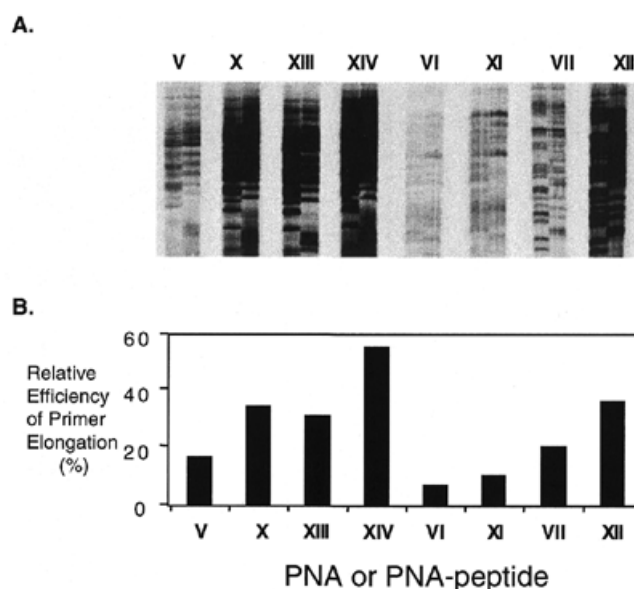
A.

B.

Figure 6. Formation of active primer–template complex as a function of PNA modification. (**A**) Strand elongation of PNA–primer–template complex evaluated by polyacrylamide gel electrophoresis. Elongation reactions were terminated by addition of ddA (left lane in each pair) or ddT (right lane in each pair). (**B**) Formation of active primer–template complex quantified by phosphorimager analysis. Efficiencies are relative to elongation by an oligonucleotide–peptide conjugate that hybridizes to almost 100% of plasmid present and represent minimum estimates for the efficiency of hybridization of PNA to plasmid. Quantitation is based on triplicate determination of strand elongation.

37°C unmodified PNA **V** allowed formation of a productive elongation complex with a second order rate constant (k_{obs}) of 1400 $M^{-1}s^{-1}$. Analogous PNAs **X**, **XIII** and **XIV**, possessing attached cationic peptides, exhibited k_{obs} values at 37°C of 2100, 2300 and 8500 $M^{-1}s^{-1}$, respectively, the latter value representing a 6-fold enhancement. The rates of hybridization were maximal when assayed at 50°C, presumably because the higher temperature increases accessibility of the target sites. At 50°C the effect of peptide attachment was greatest, with k_{obs} for **XIV** being 16-fold higher than the analogous unmodified PNA **V**. We observed similar increases in k_{obs} for hybridization of PNA–peptide **XII** relative to analogous PNA **VII** (Table 2 and Fig. 7). We also examined the effect of PNA concentration on hybridization efficiency and observed that hybridization by PNA **V** and PNA–peptide conjugate **X** showed a similar dependence on PNA concentration, with hybridization efficiency increasing as the concentration was varied from 100 nM to 1 mM (Fig. 8).

Table 2. Rate constants of association (k_{obs}) as a function of target site and temperature

PNA	k_{obs} ($M^{-1}s^{-1}$)			
	0°C	22°C	37°C	50°C
V	ND	1000	1400	1700
X	ND	1000	2100	4000
XIII	600	1000	2300	6400
XIV	500	1800	8500	27 000
VII	ND	800	1600	2100
XII	500	900	2100	4100

ND, not detectable.

Effect of mismatched bases on strand invasion

Cationic peptides should enhance binding regardless of PNA sequence and might decrease the discrimination against pairing of mismatched sequences, and a slight loss of mismatch discrimination had previously been observed for hybridization of lysine-rich polypyrimidine bis-PNAs (33). To test this hypothesis in our system, we tested PNA–peptide conjugates **XV** and **XVI** containing one or three mismatched bases, respectively, for their ability to promote strand elongation. We observed that PNA–peptide conjugate **XV**, containing one mismatch, did promote strand elongation, but elongation was 2-fold less efficient than that promoted by fully complementary PNA–peptide conjugate **X** (Fig. 9). PNA–peptide conjugate **XVI**, containing three mismatches, did not promote strand elongation. Similar results were obtained when strand elongation by unmodified PNA **V** was compared with PNAs **XVII** and **XVIII** containing one or three mismatched bases, with a single mismatch also reducing strand invasion by 2-fold. These results demonstrate that PNA–peptide conjugates retain the high level of mismatch discrimination previously reported for PNAs alone (3).

Applications of enhanced recognition by PNAs

Understanding the potential for PNAs to contribute to biotechnology and medicine requires an appreciation of the rules that govern PNA binding to complementary sequences. The
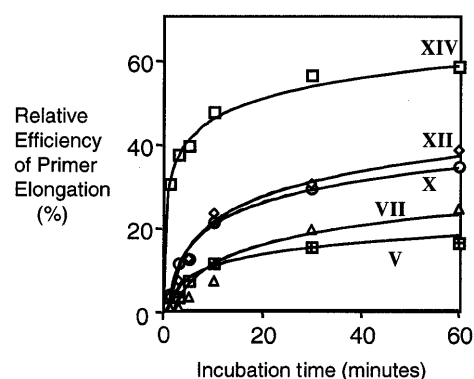

Figure 7. Dependence on incubation time of plasmid recognition by PNA **V** and PNA–peptide conjugates **VII**, **X**, **XII** and **XIV**.
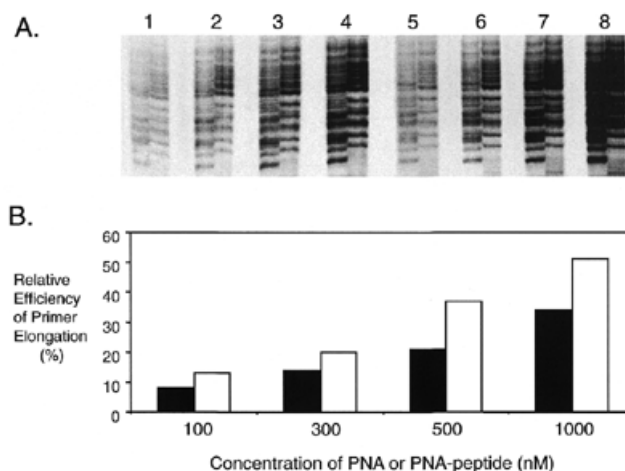

Figure 8. Dependence on PNA concentration of plasmid recognition by PNA **V** and PNA–peptide conjugate **X**. (**A**) Strand elongation evaluated by PAGE. Lanes 1–4, promotion of strand elongation by 100, 300, 500 and 1000 nM PNA **V**; lanes 5–8, promotion of strand elongation by 100, 300, 500 and 1000 nM PNA–peptide conjugate **X**. Elongation reactions were terminated by addition of ddA (left lane in each pair) or ddT (right lane in each pair). (**B**) Results in (A) quantified by phosphorimager analysis. Filled bars, PNA **V**; open bars, PNA–peptide conjugate **X**. Efficiencies are relative to elongation by an oligonucleotide–peptide conjugate that hybridizes to almost 100% of plasmid present and represent minimum estimates for the efficiency of hybridization of PNA to plasmid.
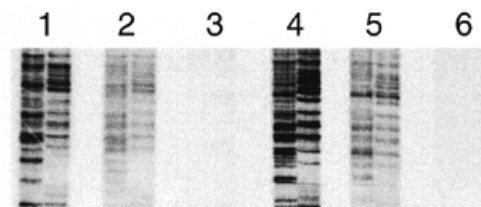

Figure 9. Effect of mismatched bases on plasmid recognition by PNA and PNA–peptide conjugates. Promotion of strand elongation by: lane 1, fully complementary PNA **V**; lane 2, singly-mismatched PNA **XVII**; lane 3, triply mismatched PNA **XVIII**; lane 4, fully complementary PNA–peptide conjugate **X**; lane 5, singly mismatched PNA–peptide conjugate **XV**; lane 6, triply mismatched PNA–peptide conjugate **XVI**. Elongation reactions were terminated by addition of ddA (left lane in each pair) or ddT (right lane in each pair).

purpose of this study was to investigate strand invasion at target sites that contain mixtures of all four bases and to examine strategies to increase the efficiency of strand invasion. We find that PNAs containing mixed base sequences as short as eight bases in length readily hybridize to inverted repeats within supercoiled plasmid DNA. These short sequences are easy to synthesize and readily soluble.

Since oligonucleotide–peptide conjugates can hybridize to sequences at or near inverted repeats without the need for PNA addition (28), it is reasonable to question whether PNAs offer significant advantages for strand invasion. One advantage is that use of PNAs allows hybridization of DNA–peptide chimeras to occur at lower temperatures, since PNAs hold open single-stranded targets at otherwise non-permissive temperatures. More importantly, PNAs also hybridize to sequences that do not contain inverted repeats and that cannot be recognized by DNA–peptide chimeras at 37°C. These results demonstrate that PNAs offer distinct advantages for strand invasion beyond those offered by DNA–peptide conjugates and expand the potential for recognition of sequences within duplex DNA by synthetic oligomers.

The inverted repeat recognized by PNAs **I–IV** originated in pBR322 and has been included in most widely used plasmid and phagemid vectors and hybridization at this sequence should be a generally useful tool for purifying plasmid DNA or for tagging plasmid DNA with fluorescent probes. Hybridization by short PNAs allows the coupling steps that had been devoted to synthesis of longer PNAs to be used more creatively for the synthesis of chimeric PNAs and bis-PNAs. Such PNA derivatives might be designed to possess additional functional properties, such as enhanced hybridization or the ability to bind to more than one DNA or RNA target simultaneously.

Strand invasion by PNAs can also occur at sequences that lack inverted repeats, even to sequences that are C/G-rich, and can be enhanced by attachment of cationic peptides. Enhanced strand invasion will facilitate applications that demand highly efficient recognition. For example, the use of PNAs as anti-gene agents *in vivo* will require binding to a high percentage of target sequences to demonstrate an unambiguous decrease in gene expression. Likewise, attempts to use PNAs to turn on gene expression will also benefit from increased hybridization efficiency. Another exciting application for enhanced strand invasion is the use of PNAs to increase the accessibility of duplex DNA to DNA–RNA chimeras designed to direct sequence-specific mutations. Currently, directed mutagenesis by these chimeras often occurs at rates of 1% or less (34) and strand invasion of target sequences by PNAs may increase the efficiency of strand correction to levels that would make this powerful technology broadly applicable to genome research. Cationic peptides have also been shown to promote delivery of attached molecules across cell membranes (35), so it is possible that a single modification might boost cellular uptake of PNAs and enhance strand invasion of genomic DNA.

Ten years of published research have demonstrated that PNAs are one of the most successful biomimetic macro-molecules designed to date and provide an important option for nucleic acid recognition. Our results suggest that strand invasion can be made more versatile and more convenient through simple chemical modifications and should encourage even wider use of PNAs for research and technology.

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